The Plant Cell, Vol. 11, 937–947, May 1999, www.plantcell.org

Three Functional Transporters for Constitutive, Diurnally Regulated, and Starvation-Induced Uptake of Ammonium into Arabidopsis Roots

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Ammonium and nitrate are the prevalent nitrogen sources for growth and development of higher plants. ^{15}N-uptake studies demonstrated that ammonium is preferred up to 20-fold over nitrate by Arabidopsis plants. To study the regulation and complex kinetics of ammonium uptake, we isolated two new ammonium transporter (*AMT*) genes and showed that they functionally complemented an ammonium uptake–deficient yeast mutant. Uptake studies with ^{14}C-methylammonium and inhibition by ammonium yielded distinct substrate affinities between $\leq$0.5 and 40 μM. Correlation of gene expression with $^{15}NH_4^+$ uptake into plant roots showed that nitrogen supply and time of day differentially regulated the individual carriers. Transcript levels of *AtAMT1;1*, which possesses an affinity in the nanomolar range, steeply increased with ammonium uptake in roots when nitrogen nutrition became limiting, whereas those of *AtAMT1;3* increased slightly, with *AtAMT1;2* being more constitutively expressed. All three ammonium transporters showed diurnal variation in expression, but *AtAMT1;3* transcript levels peaked with ammonium uptake at the end of the light period, suggesting that *AtAMT1;3* provides a link between nitrogen assimilation and carbon provision in roots. Our results show that high-affinity ammonium uptake in roots is regulated in relation to the physiological status of the plant at the transcriptional level and by substrate affinities of individual members of the *AMT1* gene family.

INTRODUCTION

Unlike most other organisms, plants are restricted to their habitats, creating potential problems when nutritional conditions become limiting. To cope with nutrient deficiencies, higher plants have developed a variety of adaptations that enable them to respond to their internal nutritional status as well as to the external availability of nutrients. In response to both, root architecture and/or transport properties are altered, leading either to a higher utilization of internalized nutrients or to enhanced nutrient acquisition at the level of nutrient mobilization or uptake (Marschner, 1995). Because nitrogen is quantitatively the most important mineral nutrient for plants, nitrogen deficiencies occur in almost all habitats at least during certain growth phases.

Among the different responses to nitrogen deficiency, altered root architecture and increased root surface have been frequently observed and seem to play key roles in the early adaptation to nitrogen deficiency (Marschner et al., 1986). A potential signal transduction intermediate has been identified recently in the MADS box gene *ANR1* from Arabidopsis that is required for the induction of lateral root growth in nitrate-rich root zones (Zhang and Forde, 1998). Thus, sensors and signal transduction cascades are required to link nutrient availability with physiological responses. On the other hand, several physiological and molecular studies have shown that nitrogen deficiency induces an enhanced capacity for nitrogen uptake by increasing NH_4^+ and NO_3^- uptake rates, which are regulated at the level of membrane transport (reviewed in von Wirén et al., 1997b). Consequently, NH_4^+ and NO_3^- transporter genes provide an important molecular target to regulate the nitrogen stress response in plants. Higher uptake rates of NH_4^+ versus NO_3^-, as found in this study with Arabidopsis, confirm a preferential uptake of the reduced nitrogen form, emphasizing that there is a larger capacity for high-affinity NH_4^+ uptake compared with NO_3^-, irrespective of the nitrogen nutritional status of the plant.

For the physiological characterization of NH_4^+ uptake in plants, an extended series of short-term uptake studies has

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been undertaken in which roots were supplied mostly with ^{13}N- or ^{15}N-labeled NH_4^+. Regarding concentration-dependent uptake kinetics, multiphasic patterns have been observed with at least two kinetically distinct components in NH_4^+ uptake: a low-affinity nonsaturable and a high-affinity saturable component (Ullrich et al., 1984; Wang et al., 1993). The first NH_4^+ transporter (*AMT*) genes were identified from yeast and Arabidopsis by functional complementation of a yeast mutant defective in high-affinity ammonium uptake (Marini et al., 1994; Ninnemann et al., 1994). A role of the plant genes in NH_4^+ nutrition is supported by the finding that NH_4^+ transporters are preferentially expressed in root hairs (Lauter et al., 1996), which make up >70% of the root surface and play a central role in nutrient uptake (Marschner, 1995).

Aside from their role in uptake of NH_4^+, NH_4^+ transporters can also act as NH_4^+ sensors. This is of particular interest because NH_4^+/NH_3 is not only the form of primary nitrogen assimilation or reassimilation but is also used as a signal for cell-to-cell communication in yeast (Palkova et al., 1997). In response to NH_4^+ availability in the growth medium, the high-affinity NH_4^+ transporter Mep2p from yeast generates a signal to regulate filamentous growth (Lorenz and Heitman, 1998). Thereby, regulation of *MEP2* in response to nitrogen is controlled by gene transcription and is distinct from that of the other two NH_4^+ transporter genes *MEP1* and *MEP3* (Marini et al., 1997). Whether similar sensing functions of NH_4^+ transporters also exist in plants remains to be shown. So far, high-affinity NH_4^+ transport has been demonstrated only for the *AMT1;1* genes from Arabidopsis and tomato (Ninnemann et al., 1994; Lauter et al., 1996). These genes belong to the same superfamily of NH_4^+ transporters as do the *MEP* genes from yeast (Marini et al., 1997). Moreover, yeast and plant NH_4^+ transporters also allowed the identification of homologs from bacteria and animals (Siewe et al., 1996; Marini et al., 1997).

To characterize the regulation of NH_4^+ transport in plants, it was our objective to isolate other members of the *AMT1* gene family from Arabidopsis and to investigate their physiological contribution to NH_4^+ uptake by roots. In this study, we report the isolation of two full-length clones, showing that the *AMT1* gene family consists of at least three members in Arabidopsis. These genes were functionally expressed in yeast for the determination of substrate affinities. In addition, their transcriptional regulation was monitored at the same as $^{15}NH_4^+$ influx into roots, allowing us to assign possible physiological functions to the three *AMT1* genes in Arabidopsis.

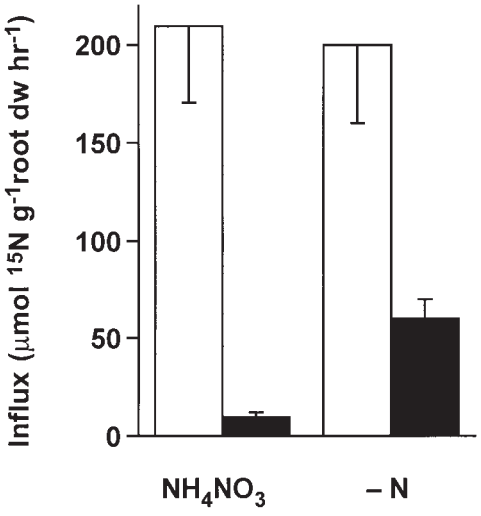

Figure 1. Influx of ^{15}N-Labeled NH_4^+ and NO_3^- in Arabidopsis Roots.

Plants were precultured hydroponically with a supply of 1 mM NH_4NO_3 or under nitrogen deficiency for 48 hr (−N). Uptake rates from 200 μM ^{15}N-labeled NH_4^+ (supplied as $[NH_4]_2SO_4$; open bars) or NO_3^- (supplied as KNO_3; filled bars) solution were measured for 5 min; $n = 6$. Error bars indicate standard deviations. dw, dry weight.

RESULTS

Preferential Ammonium Uptake by Arabidopsis Roots

To examine the physiological preference of Arabidopsis roots for NH_4^+ versus NO_3^- as a nitrogen source, we grew Arabidopsis plants hydroponically without or with a supply of 1 mM NH_4NO_3, and we measured influxes of NH_4^+ and NO_3^- after transfer to fresh nutrient solution containing either 200 μM ^{15}N-labeled NH_4^+ or NO_3^-. At an adequate nitrogen nutritional status of the plants, the NH_4^+ uptake rate exceeded that of NO_3^- by >20-fold (Figure 1), whereas in nitrogen-deficient plants, NH_4^+ influx was still fourfold higher. This relative preference of NH_4^+ uptake over NO_3^- indicates that high-affinity transport systems for both nitrogen forms are differentially regulated and that there is a larger uptake capacity for the reduced nitrogen form irrespective of the nitrogen nutritional status.

Gene Isolation of *AtAMT1;2* and *AtAMT1;3*

A cDNA library prepared from Arabidopsis plants was screened using *AtAMT1;1* as a probe (Ninnemann et al., 1994). Two homologous cDNA clones were isolated and named *AtAMT1;2* and *AtAMT1;3*. However, a comparison of the amino acid sequences to that of *AtAMT1;1* indicated rearrangements at the 5′ end of both clones. Intact full-length sequences were obtained by polymerase chain reaction (PCR) from a cDNA library by using primers annealing to the vector arms and primers specific for *AtAMT1;2* and *AtAMT1;3.* The complete open reading frames of *AtAMT1;2* and *AtAMT1;3* encode 54.9- and 55.7-kD polypeptides of 512 and 520 amino acid residues, respectively (Figure 2). Whereas

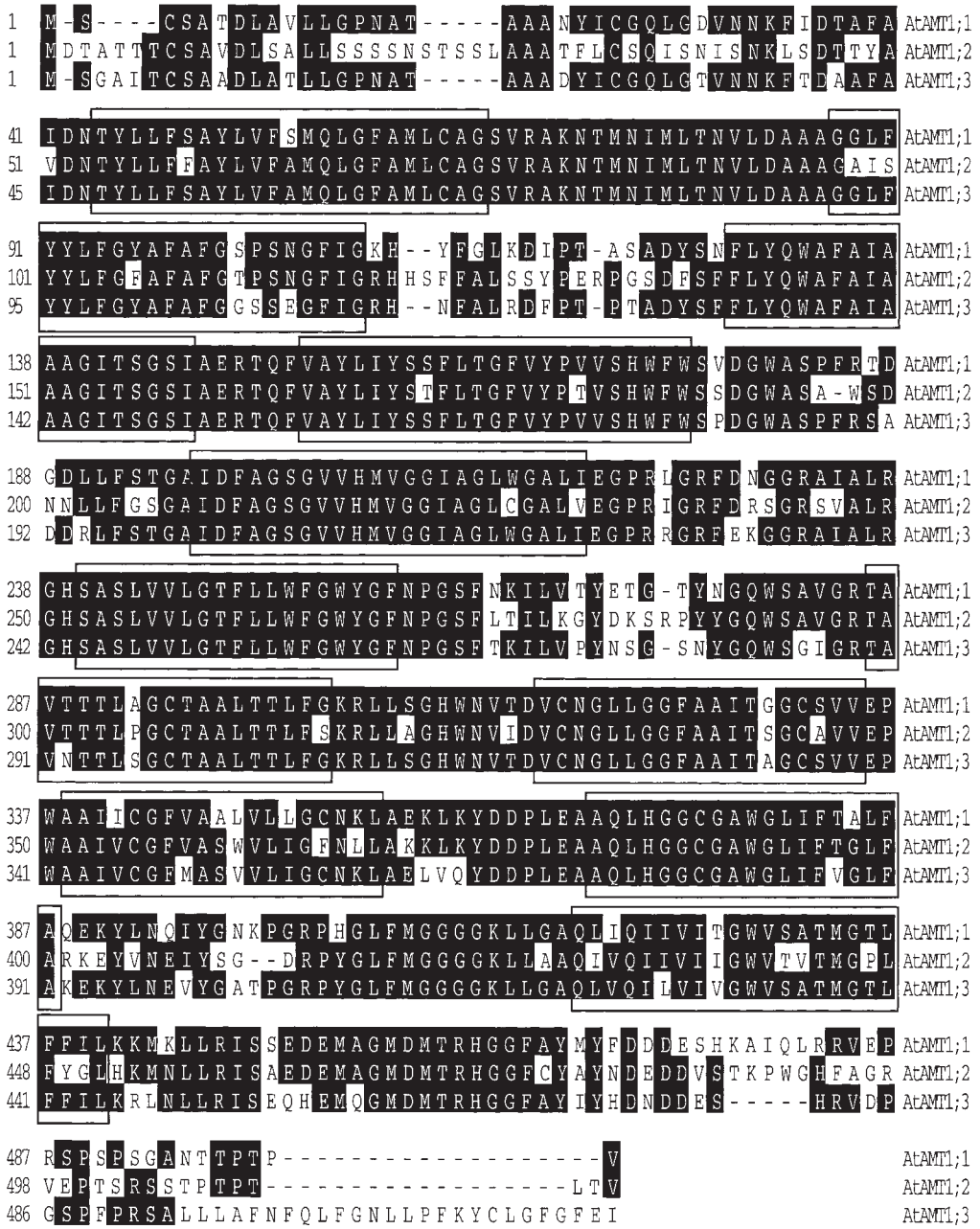

Figure 2. Amino Acid Sequence Alignment of Ammonium Transporters of the *AMT1* Family from Arabidopsis.

Identical residues are shown in black, and relative positions of transmembrane-spanning domains (Sonnhammer et al., 1998) are enclosed in boxes. Dashes indicate gaps. The GenBank accession numbers for the *AtAMT1;2* and *AtAMT1;3* cDNA sequences are AF083036 and AF083035, respectively.

AtAMT1;3 is closely related to AtAMT1;1 with 79.4% similarity at the amino acid level, AtAMT1;2 is more distantly related, with 71.5 and 67.8% similarity to AtAMT1;1 and AtAMT1;3, respectively. Interestingly, a long serine-rich domain is located in the N terminus of AtAMT1;2. Different methods predicted 11 transmembrane helices in the deduced polypeptides of all three AMT proteins (Kyte and Doolittle, 1982; Hofmann and Stoffel, 1993; Sonnhammer et al., 1998), which is in agreement with predictions made for AMT1/MEP polypeptides from tomato, rice, and yeast (Lauter et al., 1996; Marini et al., 1997; von Wirén et al., 1997a).

To estimate the total number of *AMT1* homologs in Arabidopsis, we conducted comparative DNA gel blot analysis. When genomic DNA was digested by HindIII and hybridized under low stringency with a 600-bp probe from the conserved region of *AtAMT1;1*, a maximum number of six bands appeared (Figure 3). A comparison to DNA gel blots hybridized under high-stringency conditions to full-length probes of the three *AMT* cDNAs showed that three of these six bands could be clearly assigned to *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3*, which do not contain HindIII restriction sites.

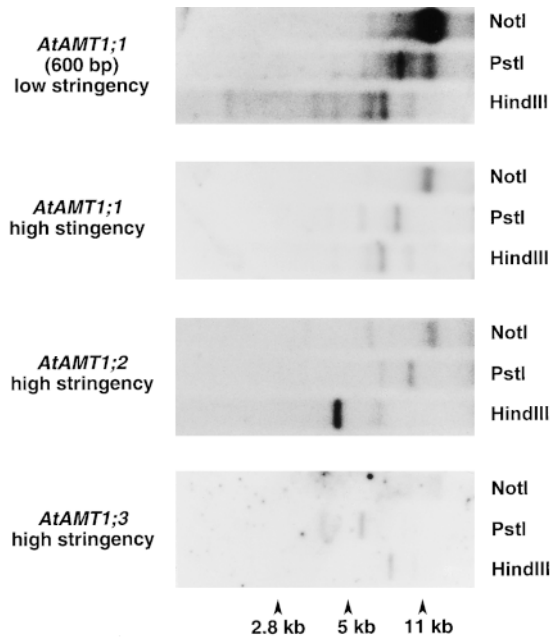

Figure 3. Comparative Gel Blot Analysis of Genomic DNA from Arabidopsis Digested with NotI, PstI, or HindIII.

After transfer to nylon membranes, DNA gel blots were hybridized with a 600-bp probe from the conserved region of *AtAMT1;1* and washed under low-stringency conditions in 2 × SSC and 0.1% SDS at 50°C. Alternatively, DNA gel blots were hybridized with full-length cDNAs from *AtAMT1;1*, *AtAMT1;2*, or *AtAMT1;3* and washed under high-stringency conditions in 2 × SSC and 0.1% SDS at 68°C. The lengths of marker fragments in kilobases are indicated below.

Thus, the other three bands most probably indicate the existence of further *AMT* homologs.

Functional Expression of *AtAMT1;1*, *AtAMT1;2,* and *AtAMT1;3* in a Yeast Mutant Defective in High-Affinity NH_4^+ Uptake

The yeast strain 31019b is defective in three endogenous NH_4^+ transporters (*mep1*, *mep2*, and *mep3*) and unable to grow on medium containing ≤5 mM NH_4^+ as the sole nitrogen source (Marini et al., 1997). Transformation of this strain with the yeast expression vector pFL61 expressing *AtAMT1;1*, *AtAMT1;2*, or *AtAMT1;3*, under the control of the constitutive yeast phosphoglycerate kinase gene promoter (Minet et al., 1992), conferred growth of 31019b down to 1 mM NH_4^+ as the sole nitrogen source (Figure 4). Thus, all three genes encode functional NH_4^+ transporters.

To determine differences in substrate affinities between the AMT proteins, we used ^{14}C-labeled methylammonium as a substrate analog, and we measured short-term uptake in transformed yeast strains. In a range of 2 to 250 μM, ^{14}C-methylammonium uptake rates were five to 20 times higher in *AMT1*-transformed cells than in cells containing the vector alone, and concentration-dependent uptake rates in all three *AMT1* transformants followed saturable kinetics (Figures 5A to 5C). Determination of affinity constants after nonlinear curve fitting and subtraction of endogenous uptake activities in vector-transformed cells showed that AtAMT1;1 displayed the highest affinity (K_m = 8 μM), followed closely by AtAMT1;3 (K_m = 11 μM) and finally by AtAMT1;2, whose affinity was considerably lower (K_m = 24 μM) (Figures 5A to 5C). Because the affinities for methylammonium do not necessarily reflect the affinity for ammonium (Venegoni et al., 1997), competition studies were performed with varying NH_4^+ concentrations. At a methylammonium concentration corresponding to the K_m of each AMT1 transporter, a 50% inhibition by NH_4^+ was found at ≤0.5 μM NH_4^+ for AtAMT1;1 but between 25 and 40 μM for AtAMT1;2 and AtAMT1;3 (Figure 5D). These competition studies showed that the three NH_4^+ transporters possess distinct substrate affinities, allowing the plant to take up external NH_4^+ over a wide concentration range. In contrast to uptakes in AtAMT1;1, a 50% inhibition of methylammonium uptake in AtAMT1;2 and AtAMT1;3 transformants was seen at equal or higher concentrations of NH_4^+ relative to methylammonium. Thus, AtAMT1;2 and AtAMT1;3 provide transport systems of minor selectivity.

Organ-Dependent Expression of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3*

Total RNA extracted from stems, source leaves, sink leaves, and buds of plants grown for 5 weeks in soil culture in a greenhouse and root RNA extracted from plants grown hydroponically under axenic conditions was hybridized to

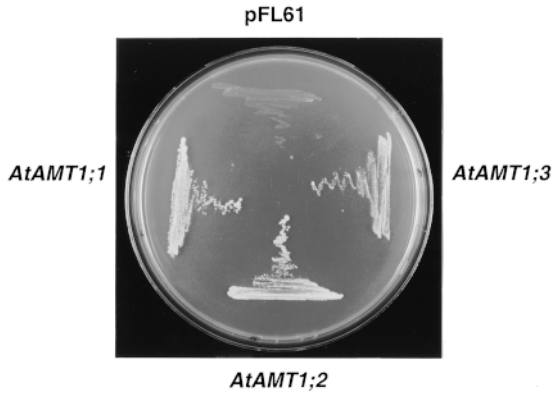

Figure 4. Growth Test on Minimal Medium Containing 1 mM NH_4^+ as the Sole Nitrogen Source.

The yeast strain 31019b (*mep1 mep2 mep3 ura3*; Marini et al., 1997) was transformed with the yeast expression vector pFL61 alone (Minet et al., 1992) or pFL61 harboring the coding sequences of *AtAMT1;1*, *AtAMT1;2*, or *AtAMT1;3.*

gene-specific probes for all three *AMT*s. *AtAMT1;1* transcripts were found in all organs examined (Figure 6). In contrast, expression of *AtAMT1;2* was mainly confined to roots with faint signals in stems and leaves, whereas under the conditions described, *AtAMT1;3* expression seemed to be exclusively restricted to roots.

Correlation between $^{15}NH_4^+$ Influx and *AMT1* Gene Expression in Roots

Because all three *AMT1* genes were strongly and preferentially expressed in roots and because the affinities for NH_4^+ of the AMT1 gene products differed widely, the question arose whether they play different physiological roles in roots. To examine a possible contribution of each single AMT to overall NH_4^+ uptake by roots, plants were grown under conditions that modulate NH_4^+ uptake rates, and *AMT1* gene expression in roots was determined simultaneously with short-term $^{15}NH_4^+$ uptake rates.

Influence of the Light–Dark Cycle

When plants were grown for 6 weeks on NH_4NO_3-containing nutrient solution, short-term $^{15}NH_4^+$ influx was low at the beginning of illumination (Figure 7A). However, toward the end of the light period, $^{15}NH_4^+$ influx increased by a factor of 3, which was followed by a sharp decrease at the beginning of darkness. A corresponding threefold increase until the end of the light period was also observed for transcript levels of *AMT1;3* (Figure 7B). Although expression of *AtAMT1;1* and *AtAMT1;2* was also higher during the daytime (1.5 and 1.7 times, respectively), correlation to NH_4^+ influx was much weaker. Therefore, it is suggested that the enhanced NH_4^+ influx at the end of the photoperiod was mainly brought about by transcriptional upregulation of *AtAMT1;3.*

Influence of Nitrogen Deficiency

Monitoring $^{15}NH_4^+$ influx of plants adequately supplied with nitrogen after transfer to nitrogen-free nutrient solution showed that $^{15}NH_4^+$ influx steeply increased, resulting in a peak after 48 to 72 hr, irrespective of whether plants were precultured with KNO_3 (data not shown) or NH_4NO_3 (Figure 8A). The increase in NH_4^+ influx coincided with an increase in *AtAMT1;1* transcript levels in roots, which accumulated fivefold relative to nonstarved plants within 72 hr (Figure 8B). Whereas *AtAMT1;2* did not show any significant change, mRNA expression of *AtAMT1;3* increased by approximately twofold. The enhanced gene expression of *AtAMT1;1* and to a minor extent also of *AtAMT1;3* suggested that their gene products mainly contribute to increased NH_4^+ influx under nitrogen deficiency.

Influence of Change of the Nitrogen Source

To differentiate between the effects of nitrogen form and nitrogen supply, plants were precultured in the presence of NH_4NO_3 and then transferred to medium containing NO_3^- as the sole nitrogen source. As with the transfer to nitrogen-free medium, $^{15}NH_4^+$ influx steeply increased within 72 hr (Figure 9A). However, none of the three *AMT* genes responded with increased transcript levels (Figure 9B), pointing to the possiblity that increased $^{15}NH_4^+$ influx after transfer to NO_3^- could be due to regulation at the post-transcriptional level. Indeed, all three *AMT* genes were expressed during the experiment, pointing to a derepression in post-transcriptional regulation after the removal of NH_4^+ rather than an induction of *AMT* expression as observed under nitrogen deficiency. Alternatively, it is also possible that enhanced $^{15}NH_4^+$ influx was mediated by NH_4^+ transport proteins other than AtAMT1;1, AtAMT1;2, and AtAMT1;3.

DISCUSSION

The *AMT1* Multigene Family in Plants

Several points of evidence indicate that proteins of the *AMT1* gene family act as functional NH_4^+ transporters in plants. First, *AMT1* genes belong to the *MEP*/*AMT1* superfamily of eukaryotic and prokaryotic NH_4^+ transporter genes, and for the yeast genes, functional knockouts have already been characterized (Marini et al., 1994, 1997). Second, *AMT1;1* genes from tomato and Arabidopsis conferred high-affinity NH_4^+ uptake to yeast mutants defective in NH_4^+

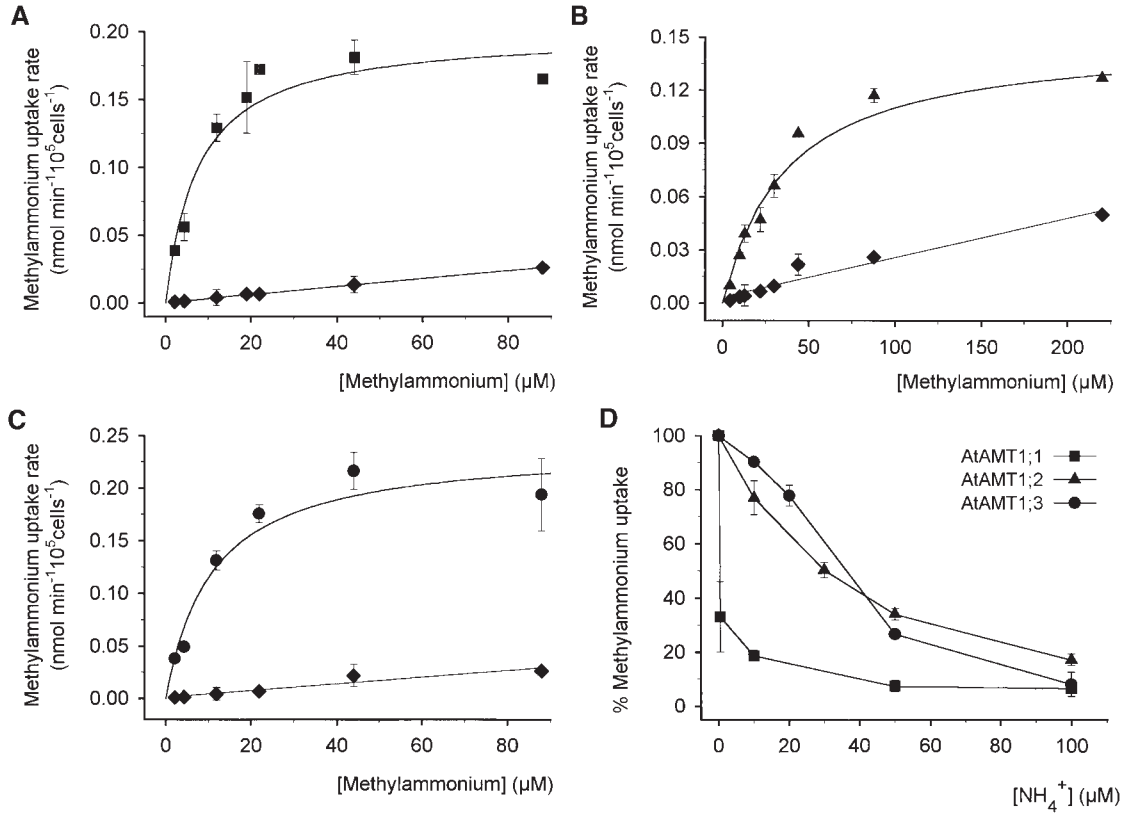

Figure 5. Kinetic Analysis of AtAMT1;1, AtAMT1;2, and AtAMT1;3 in Transformed Yeast.

(A) to **(C)** Concentration-dependent kinetics of ^{14}C-methylammonium uptake by yeast strain 31019b (*mep1 mep2 mep3 ura3*; Marini et al., 1997) transformed with pFL61 alone (filled diamonds) or pFL61 harboring the following coding sequences: *AtAMT1;1* (filled squares **[A]**), *AtAMT1;2* (filled triangles **[B]**), or *AtAMT1;3* (filled circles **[C]**).

(D) The influence of increasing NH_4^+ concentrations on ^{14}C-methylammonium uptake rates. Inhibition of ^{14}C-methylammonium uptake was measured at the corresponding K_m of each AMT. 100% uptake corresponds to 0.12, 0.10, and 0.24 nmol of methylammonium min^{-1} per 10^5 cells for AtAMT1;1, AEAMT1;2, and AtAMT1;3, respectively. Error bars indicate standard errors from uptake measurements with at least three independent transformants. If not visible, error bars are within the plot symbols.

transport (Ninnemann et al., 1994; Lauter et al., 1996), which strongly suggests that AMT proteins are also targeted to the plasma membrane in plants. Moreover, biochemical characteristics of AMT-mediated transport in yeast, such as energy dependence, pH optimum, and inhibitor sensitivity to K^+ (Ninnemann et al., 1994), reflect those of NH_4^+ uptake by intact plant roots (Wang et al., 1993, 1996). Finally, expression of *AMT1;1* in tomato was found preferentially in root hairs, strongly supporting a role for *AMT* genes in primary NH_4^+ acquisition from the growth medium (Lauter et al., 1996).

After isolation of *AtAMT1;1* (Ninnemann et al., 1994), we now show that *AMT1* is a multigene family consisting of at least three members in Arabidopsis (Figure 2). Full-length cDNAs were isolated for *AtAMT1;2* and *AtAMT1;3*, with *AtAMT1;3* being more closely related to *AtAMT1;1.* However, because low-stringency DNA gel blot analysis with a conserved *AMT1* sequence resulted in a maximum of six bands (Figure 3), additional members of the *AMT1* gene family might be expected. The existence of several NH_4^+ transporter genes in Arabidopsis suggests that membrane transport of NH_4^+ is a highly regulated process and emphasizes the importance of NH_4^+ as a main mineral nutrient in plants. This is supported by the finding that NH_4^+ is the preferential form for nitrogen uptake at low external concentrations (Figure 1). Therefore, we hypothesized that transporters of the *AMT1* gene family are responsible for the large capacity of high-affinity NH_4^+ uptake in plants, and we defined their function in NH_4^+ transport.

Apart from a pivotal role in nutrient uptake, AMT proteins could play a role in nutrient sensing required for root growth, as in the case of the related NH_4^+ transporter Mep2p from yeast (Lorenz and Heitman, 1998). Thus far, no structural features were observed in the AMT1 sequences that could point to a role in nutrient sensing such as extended hydro-

philic domains for protein–protein interactions, as found in RGT2/SNF3 (Özcan et al., 1996). However, because Mep2p of the AMT1/MEP superfamily acts as a sensor without showing particular structural features (Lorenz and Heitman, 1998), it is uncertain whether NH_4^+-sensing functions can be identified at a structural level.

Three *AMT1* Transporters Expressed in Roots Show Different Affinities for Ammonium

To elucidate the possible physiological roles of AtAMT1;1, AtAMT1;2, and AtAMT1;3 at a functional and regulatory level, we expressed all three full-length clones in yeast to determine substrate affinities; on the other hand, *AMT1* gene expression was related to root NH_4^+ uptake activity in different physiological conditions. Determination of affinity constants for ^{14}C-methylammonium uptake by AtAMT1;1, AtAMT1;2, and AtAMT1;3 in yeast and uptake inhibition by NH_4^+ show that these high-affinity NH_4^+ transporters are differently adapted to transport at low concentrations of external NH_4^+. Although the calculated transport affinities ranging from ⩽0.5 to 40 μM might still be subject to environmental modifications, they indicate that optimum transport capacities of the three AtAMT1 transporters cover precisely the range of NH_4^+ concentrations typically found in soils (Marschner, 1995).

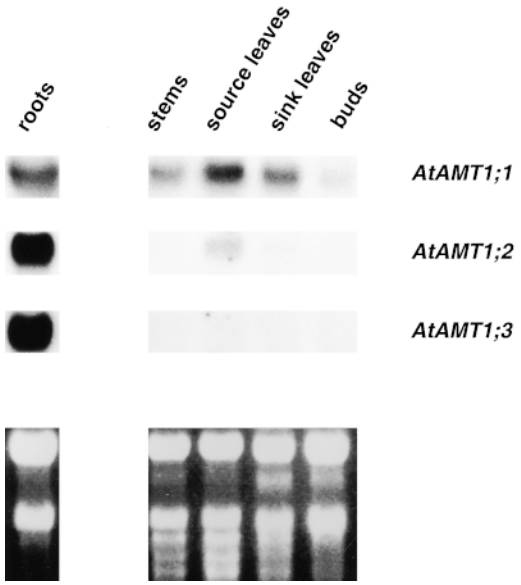

Figure 6. Organ-Dependent Expression of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* in Roots, Stems, Mature Leaves, Young Leaves, and Buds of Arabidopsis.

Plants were grown under sterile conditions in liquid Murashige and Skoog medium in the growth chamber for root RNA extraction (left) or in soil culture in the greenhouse for RNA extraction from shoot organs. The gels below are shown as a loading control.

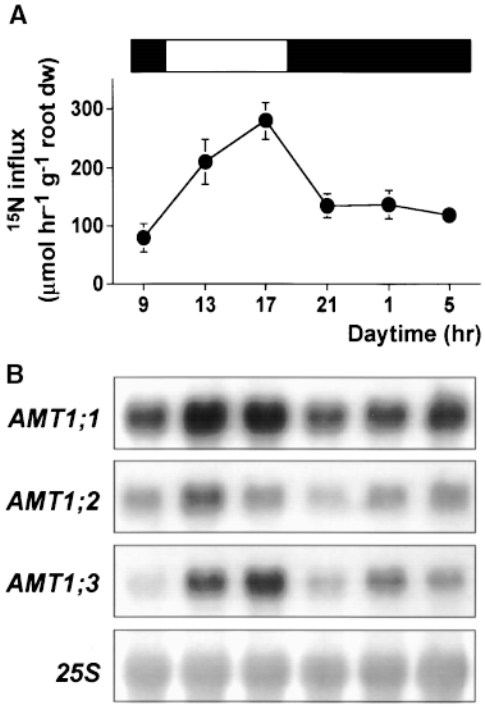

Figure 7. Correlation between NH_4^+ Influx and *AMT* Gene Expression at Different Times of Day.

(A) Time-of-day–dependent influx of $^{15}NH_4^+$ in Arabidopsis roots grown on 1 mM NH_4NO_3. Root uptake rates in 200 μM $^{15}NH_4^+$ were measured for 5 min; $n = 12$. The white bar indicates light and the black bar indicates dark conditions for plant growth. Error bars indicate standard deviations. dw, dry weight.

(B) RNA gel blot analysis of the expression of *AtAMT1;1*, *AtAMT1;2*, or *AtAMT1;3.* Total root RNA was extracted from plants and used as given for $^{15}NH_4^+$ influx studies. 25S indicates the rRNA loading control.

In yeast, two of the three *MEP* genes encode high-affinity NH_4^+ transporters (K_i of NH_4^+ for ^{14}C-methylammonium uptake 5 to 10 μM and 1 to 2 μM for Mep1p and Mep2p, respectively), whereas Mep3p possesses a much lower affinity of ⩾1.4 mM (Marini et al., 1997). Whether, similar to the Mep proteins, low-affinity transporters are also included in the *AMT1* gene family of Arabidopsis remains to be demonstrated. Theoretically, entry of NH_4^+ could also be mediated as a side activity of less specific cation channels, in particular K^+ transporters and channels (Schachtman and Schroeder, 1994; White, 1996).

In uptake studies with whole plants, it has been shown that NH_4^+ has an inhibitory effect on low-affinity K^+ uptake with the exception of growth conditions under K^+ deficiency (Wang et al., 1996). This might be due to an NH_4^+-induced membrane depolarization, which generally decreases ion

influx (Ayling, 1993), rather than through direct competition between the two ions, reinforcing the anticipation that the entry pathways of both ions might be independent. On the other hand, in patch–clamp studies with root hairs of wheat, a 15% conductance for NH_4^+ has been measured for inward-rectifying K^+ channels (Gassmann and Schroeder, 1994), which might contribute to low-affinity NH_4^+ uptake in plant roots (Gassmann et al., 1993). Because NH_4^+ entry by K^+ channels requires NH_4^+ concentrations above 0.1 to 1 mM (White, 1996) and average annual soil concentrations rarely rise beyond 50 μM (Marschner, 1995), the ecological significance of this transport path for field-grown plants might be restricted to periods of high nitrogen mineralization or NH_4^+ fertilizer application. In addition, NH_4^+ might be taken up into cells via diffusion of NH_3, a process that gains

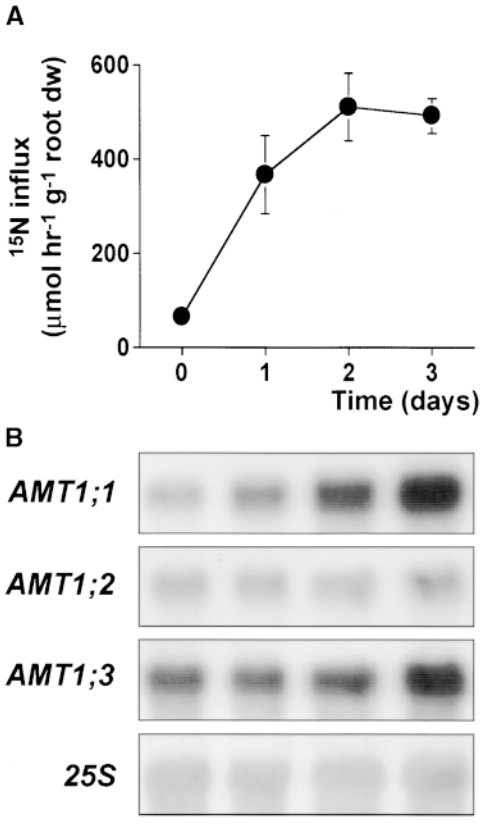

Figure 8. Correlation between NH_4^+ Influx and *AMT* Gene Expression after Subjecting Roots to Nitrogen Deficiency.

(A) $^{15}NH_4^+$ influx in Arabidopsis roots after transfer of hydroponically grown plants from 1 mM NH_4NO_3 to nitrogen-free nutrient solution. Uptake rates in 200 μM $^{15}NH_4^+$ were measured for 5 min; $n = 6$. Error bars indicate standard deviations. dw, dry weight.

(B) RNA gel blot analysis of expression of *AtAMT1;1*, *AtAMT1;2*, or *AtAMT1;3*. Total root RNA was extracted from plants as used for $^{15}NH_4^+$ influx studies. 25S indicates rRNA loading control.

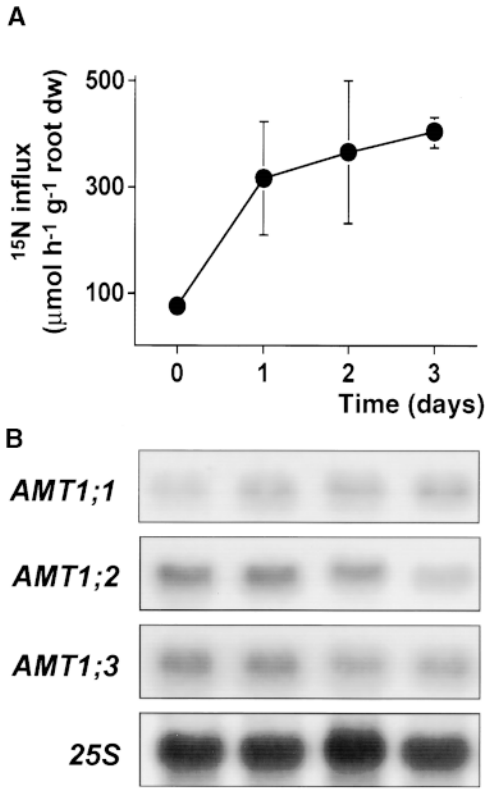

Figure 9. Correlation between NH_4^+ Influx and *AMT* Gene Expression after Transfer of Roots to NO_3^- as the Sole Nitrogen Source.

(A) $^{15}NH_4^+$ influx in Arabidopsis roots after transfer of hydroponically grown plants from 1 mM NH_4NO_3 to 1 mM KNO_3. Uptake rates in 200 μM $^{15}NH_4^+$ were measured for 5 min; $n = 6$. Error bars indicate standard deviations. dw, dry weight; h, hour.

(B) RNA gel blot analysis for the expression of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3*. Total root RNA was extracted from plants as used for $^{15}NH_4^+$ influx studies. 25S indicates rRNA loading control.

in importance with increasing external pH. However, to significantly contribute to plant nitrogen nutrition, these pathways require external NH_4^+ concentrations in the millimolar range.

AMT1 gene expression is organ dependent and differentially regulated for *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3*. All three genes are highly expressed in roots (Figure 6), but expression of *AtAMT1;1* and *AtAMT1;2* was also found in shoot organs, being highest in mature leaves. A physiological role of NH_4^+ transporters in leaves is evident for NH_4^+ import from the vascular system across the mesophyll plasma membrane, because NH_4^+ concentrations in the xylem can rise to 2.6 mM under exclusive NH_4^+ supply or even up to 300 μM in the absence of supplied NH_4^+ (Cramer and Lewis, 1993). On the other hand, NH_4^+ transporters in mesophyll cells might be involved in the retrieval of photorespira-

tory NH_3/NH_4^+. Even under ambient CO_2 concentrations, the loss of photorespiratory NH_3 in mitochondria (Keys et al., 1978) can lead to lethality or at least to a dramatic increase in leaf ammonia concentrations if reassimilation of NH_3 is absent or inhibited, as is the case for photorespiratory mutants from barley and Arabidopsis (Somerville and Ogren, 1980; Wallsgrove et al., 1987). Because photorespiratory NH_3 is likely to be reprotonated during passage to the cytosol or when released to the leaf apoplast, a reimport via NH_4^+ transporters such as the *AMT1* gene family might be required. Therefore, it will be of particular interest to determine their cellular localization and regulation under different photorespiratory conditions.

Evidence for the Role of *AtAMT1;1* in Deficiency-Induced and *AtAMT1;3* in Diurnally Regulated Ammonium Uptake

High expression levels of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* in roots (Figure 6) suggested that all three *AMT* genes play a role in root NH_4^+ uptake from the growth medium. Moreover, evidence for different physiological functions of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* came from the observations that diurnal variations in $^{15}NH_4^+$ influx correlated with transcript levels of *AtAMT1;3*, whereas enhanced $^{15}NH_4^+$ influx after plant transfer to nitrogen-free nutrient solution closely correlated with transcriptional upregulation of *AtAMT1;1* (Figures 7 and 8). Therefore, we concluded that AtAMT1;1 is mainly responsible for the enhanced capacity for NH_4^+ uptake under nitrogen deficiency. This is of particular physiological significance because AtAMT1;1 is the transporter with the highest substrate affinity, indicating that high nitrogen demand triggers induction of the transporter, which allows most efficient uptake even from external concentrations in the nanomolar range. An additional contribution of AtAMT1;3 to NH_4^+ uptake under these conditions is indicated by the smaller but significant increase in *AtAMT1;3* transcript levels.

On the other hand, *AtAMT1;3* transcription was strongly induced at the end of the light period, which usually coincides with high carbohydrate levels in roots required for NH_4^+ assimilation (Kerr et al., 1985). This suggests that transcriptional regulation of *AtAMT1;3* might also be controlled by the availability of carbon skeletons in roots, thereby providing a physiological link between nitrogen and carbon metabolism in plants. However, it cannot be excluded that *AtAMT1;3* is linked to diurnal regulation mediated by the circadian clock or by changes in any other metabolites. This might also be the case for both *AtAMT1;1* and *AtAMT1;2* transcription, which slightly decreased during the dark period (Figure 7B).

Among all three *AMT* genes, *AtAMT1;2* showed most stable expression levels in all experiments, possibly pointing to a role in constitutive high-affinity uptake of NH_4^+ at external concentrations in the micromolar to millimolar range. However, because only a few physiological conditions have been investigated, these studies provide correlative evidence for different physiological roles of the individual *AMT* genes on the basis that further *AMT* homologs did not or weakly cross-hybridized with *AtAMT1;1* to *AtAMT1;3.* To obtain direct evidence for the physiological role of the individual NH_4^+ transporter genes in Arabidopsis, we identified knockout mutants by using an approach similar to that used to identify an insertion in K^+ channel genes (Gaymard et al., 1998; Hirsch et al., 1998). A subsequent combination of the K^+ and NH_4^+ transport mutants might then allow a complete overview of the genes involved in both low- and high-affinity NH_4^+ transport to be obtained. So far, the present data could demonstrate that both adapted substrate affinities and transcriptional regulation of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* allow the plant to respond differentially to varying nutritional conditions in the environment as well as within the plant.

METHODS

Library Screening and DNA and Sequence Analyses

A λ ZAPII cDNA library made from *Arabidopsis thaliana* seedlings (Minet et al., 1992) was screened using *AtAMT1;1* (Ninnemann et al., 1994) as probe. After in vivo excision, the sequences of isolated clones were determined on both strands. To verify 5′ sequences of *AtAMT1;2* and *AtAMT1;3*, polymerase chain reactions (PCRs) were performed with Pfu polymerase (Stratagene, La Jolla, CA) with an Arabidopsis cDNA library as template (Minet et al., 1992), using reverse primers specific for *AtAMT1;2* (5′-GCGGCGAGGGAAGATGTTGAGTTA-3′) and *AtAMT1;3* (5′-AGCGGCCGCGATTGCGAACGCCCA-3′) and a forward primer carrying the vector arm sequence (5′-TATTTTAGCGTAAAGGATGGGGAAA-3′). DNA sequences of *AtAMT1;2* and *AtAMT1;3* have DDBJ/EMBL/GenBank accession numbers AF083036 and AF083035, respectively.

Isolation of genomic DNA from seedlings and of RNA from different organs of Arabidopsis plants as well as DNA gel blot and RNA analyses were essentially performed as given in Ninnemann et al. (1994). For this purpose, Arabidopsis ecotype C24 plants were grown either in axenic culture on Murashige and Skoog medium (Difco, Augsburg, Germany) supplemented with 2% sucrose, as described by Touraine and Glass (1997), or in soil culture in the greenhouse.

Yeast Transformation and Uptake Measurements

To clone *AtAMT1;2* and *AtAMT1;3* into the NotI site of the yeast expression vector pFL61, we designed specific primers that included Bsp120I restriction sites, and PCR reactions were run using Pfu polymerase on the Arabidopsis cDNA library. Primers used for *AtAMT1;2* and *AtAMT1;3* are as follows: 5′-TCTCCCTCGGGCCCTCTCCACCATGGACACCGC-3′ and 5′-GACTCGTTTGGGCCCACTCAATTCTCC-3′; and 5′-TTTGGCGGGCCCATGTCAGGAGCTATAACATGCTCTGCGGCC-3′ and 5′-CAAACCGGGCCCTCCAAATATTTATATTTCAAAACCAAAGCCC-3′, respectively.

The yeast strain 31019b (*mep1*Δ *mep2*Δ::*LEU2 mep3*Δ::*KanMX2 ura3*; Marini et al., 1997) was transformed with pFL61 harboring the

cDNA sequence of *AtAMT1;1* or PCR products of *AtAMT1;2* and *AtAMT1;3*, according to Dohmen et al. (1991). Yeast transformants were selected on nitrogen-free medium (NAA; Difco) supplemented with 0.5 mM $(NH_4)_2SO_4$ and 2% glucose. For uptake measurements, yeast cells were grown to the logarithmic phase in NAA medium supplemented with 2% glucose and 500 μg/mL L-proline. Cells were harvested at $OD_{620\,nm}$ of 0.5 to 0.7, washed, and resuspended in 20 mM sodium phosphate buffer, pH 7, to a final $OD_{620\,nm}$ of 8. Five minutes before the uptake measurement, cells were supplemented with 100 mM glucose and incubated at 30°C. To start the reaction, we added 100 μL of this cell suspension to 100 μL of the same buffer containing different concentrations of ^{14}C-methylammonium (2.11 Gbq/mmol; Amersham), and after 0.5, 1, 2, and 4 min, aliquots were withdrawn, diluted in 4 mL of ice-cold sodium phosphate buffer containing 100 mM methylammonium, and filtered through glass fiber filters (GF/C; Whatman International Ltd., Maidstone, UK). Filters were washed twice with 4 mL of water and analyzed by liquid scintillation spectrometry. For inhibition studies, different concentrations of NH_4^+ as $(NH_4)_2SO_4$ were added to the ^{14}C-methylammonium solution.

^{15}N-Uptake Studies

Arabidopsis seeds (ecotype Columbia C24) were germinated, and seedlings were grown hydroponically for 18 days under sterile conditions (Figures 7 and 8) according to the protocol described by Touraine and Glass (1997), with the exception that hydroponic vessels harbored seven to 10 seedlings on 60 mL of the following nutrient solution: 1 mM NH_4NO_3, 1 mM $CaSO_4$, 1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 50 μM NaFeEDTA, 50 μM H_3BO_3, 12 μM $MnCl_2$, 1 μM $CuCl_2$, 1 μM $ZnCl_2$, 30 nM $(NH_4)_6Mo_7O_{24}$, 10 g/L sucrose, and 0.5 g/L Mes. The pH was adjusted to 5.7. Nitrogen-free or NO_3^- solutions used in the experiments had the same composition as the NH_4NO_3 solution used for growth, except that 1 mM NH_4NO_3 was omitted or replaced by 1 mM KNO_3, respectively. To avoid depletion, the nutrient solution was renewed twice during the first 10 days of growth and then daily in the week preceding and during the experiments.

Plants were cultivated in a growth chamber at 60% relative humidity, with a light intensity of 200 μmol m^{-2} sec^{-1} and a day–night temperature regime of 8 hr at 22°C and 16 hr at 20°C, respectively. To cultivate plants in the absence of sucrose (Figure 6), we grew plants under nonsterile conditions and a higher light intensity of 400 μmol m^{-2} sec^{-1} and a day–night temperature regime of 16 hr at 24°C and 8 hr at 20°C, respectively. To support the plants, the bottoms of Eppendorf tubes were cut and replaced by stainless steel mesh. The tubes were filled with sand and placed in plastic holders in a polystyrene raft floating on nutrient solution in a 10-L cuvette. Seeds were germinated directly on prewetted sand; after 2 weeks, plants were transferred to complete nutrient solution containing 1 mM NH_4NO_3 as the nitrogen source. Influx of $^{15}NH_4^+$ in roots was determined after transferring the plants first to 0.1 mM $CaSO_4$ for 1 min, then to nutrient solution containing 0.2 mM ^{15}N-labeled NH_4^+ (99 atom% ^{15}N) for 5 min, and finally to 0.1 mM $CaSO_4$ for 1 min. The influx solution was the same as used for plant growth except that 1 mM NH_4NO_3 was replaced by 0.1 mM $(^{15}NH_4)_2SO_4$. Roots were separated from shoots and dried for 48 hr at 70°C. ^{15}N contents were determined by mass spectrometry using a continuous-flow isotope ratio mass spectrometer coupled with an elemented analyzer (model ANCA-MS; Europa Scientific, Crewe, UK; Clarkson et al., 1996).

RNA was extracted from roots according to Lobreaux et al. (1992). Ten or 20 μg of total RNA was electrophoresed through formaldehyde agarose gels and transferred to nylon membranes (Hybond N; Amersham). Prehybridizations were performed for 4 hr at 42°C in 50% formamide, 4 × SSPE (1 × SSPE is 0.15 M NaCl, 15 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 1% sarkosyl, 10% dextran sulfate, and 100 μg/mL denatured salmon sperm DNA. Hybridizations were achieved overnight at 42°C in the same buffer containing the ^{32}P-labeled cDNA probe representing full-length cDNAs. Filters were washed twice in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS and twice in 0.1 × SSC and 0.1% SDS for 15 min at 42°C. A 25S rRNA cDNA probe was used as a reference for relative quantifications conducted with a PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA). All correlative experiments were conducted at least two times independently and yielded similar results.

ACKNOWLEDGMENTS

We thank Wolfgang Jost (ZMBP, Tübingen, Germany) and Pascal Tillard (Biochimie et Physiologie Moléculaire des Plantes, Montpellier, France) for excellent technical assistance and Bruno André (Université Libre de Bruxelles, Brussels, Belgium) for providing the yeast mutant. This work was supported by the European Community BIOTECH4 program EURATINE.

Received October 9, 1998; accepted February 19, 1999.

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